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Activation of the Lck Tyrosine Protein Kinase by the Herpesvirus Saimiri Tip Protein Involves Two Binding Interactions

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The Tip protein of Herpesvirus saimiri strain 484C binds to and activates the Lck tyrosine protein kinase. Two sequences in the Tip protein were previously shown to be involved in binding to Lck. A proline-rich region, residues 132–141, binds to the SH3 domain of the Lck protein. We show here that the other Lck-binding domain, residues 104–113, binds to the carboxyl-terminal half of Lck and that this binding does not require the Lck SH3 domain. Mutated Tip containing only one functional Lck-binding domain can bind stably to Lck, although not as strongly as wild-type Tip. Interaction of Tip with Lck through either Lck-binding domain increases the activity of Lck *in vivo.* Simultaneous binding of both domains is required for maximal activation of Lck. The transient expression of Tip in T cells was found to stimulate both Stat3-dependent and NF-AT-dependent transcription. Mutant forms of Tip lacking one or the other of the two Lck-binding domains retained the ability to stimulate Stat3-dependent transcription. Tip lacking the proline-rich Lck-binding domain exhibited almost wild-type activity in this assay. In contrast, ablation of either Lck-binding domain abolished the ability of Tip to stimulate NF-AT-dependent transcription. Full biological activity of Tip, therefore, appears to require both Lck-binding domains. © 2000 Academic Press

INTRODUCTION

New World monkeys (Meléndez *et al.,* 1971) or New Zealand white rabbits (Daniel *et al.,* 1974) infected with Herpesvirus saimiri (HVS) rapidly succumb to lymphoma or leukemia of T-cell origin. Human peripheral blood lymphocytes can also be infected and transformed *in vitro* by group C strains of this virus (Biesinger *et al.,* 1992; Medveczky *et al.,* 1993). Cells that are transformed by HVS of strain 484C are phenotypically mature peripheral T cells of predominantly the CD8 single-positive lineage (Medveczky *et al.,* 1993). HVS-transformed cells have been shown to express and secrete interleukin-2 in the absence of T-cell antigen receptor stimulation (Chou *et al.,* 1995). Work with two strains of Herpesvirus saimiri (484C and 488C) has demonstrated that the left end of the viral genome is required for the transforming potential, but dispensable for viral replication (Desrosiers *et al.,* 1985; Koomey *et al.,* 1984; Medveczky *et al.,* 1993). Two open reading frames have been identified within this region that are expressed from a single, bicistronic mRNA (Murthy *et al.,* 1989). One of these open reading frames encodes the tyrosine kinase interacting protein, or Tip, so named because it binds tightly to the Lck tyrosine protein kinase (Biesinger *et al.,* 1995; Lund *et al.,* 1996). The other open reading frame encodes a protein called STP (for saimiri transforming protein) (Murthy *et al.,* 1989). Both Tip and STP are necessary for the transforming ability of strains that express both proteins (Duboise *et al.,* 1998a; Medveczky *et al.,* 1993). Our investigation of the interaction of Tip with the Lck kinase are reported here.

Lck is a member of the Src family of cytoplasmic tyrosine protein kinases whose expression is largely restricted to lymphoid cells (Sefton, 1991). Mice lacking an Lck gene produce only 10% as many T cells as do normal mice (Molina *et al.,* 1992). Additionally, Lck is important in signaling from the T-cell antigen receptor in mature T cells. A derivative of the Jurkat cell line that has lost expression of Lck, JCaM1, is unresponsive to stimulation through the T-cell receptor (Straus and Weiss, 1992). Because reintroduction of Lck restores signaling, it is clear that it is the absence of Lck that is responsible for the signaling defect in these cells. The T-cell receptor and the ZAP-70 tyrosine protein kinase are both likely to be direct substrates of Lck. Finally, expression of an activated form of Lck in T-cell lines leads to spontaneous, antigen-independent production of interleukin-2 (Luo and Sefton, 1992). This indicates that Lck-activated

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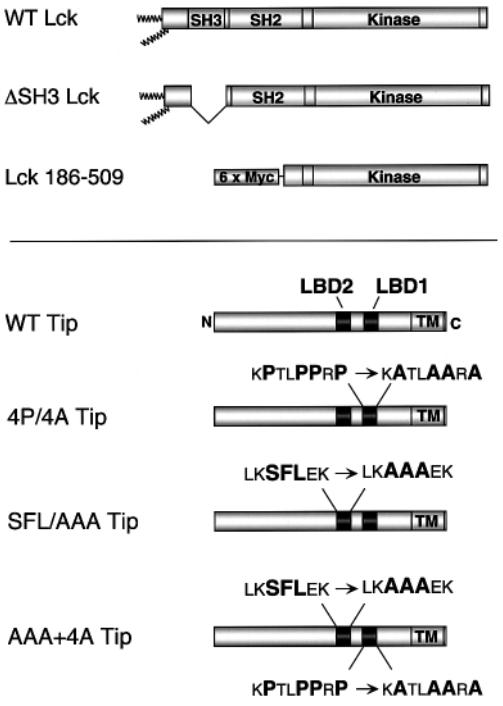

FIG. 1. Lck and Tip constructs used in this study. Indicated for Lck are the N-terminal membrane-binding region containing two sites of fatty acylation, the SH3 domain, the SH2 domain, the catalytic domain, and the carboxyl-terminal regulatory domain. Indicated in the diagram of Tip are the presumptive transmembrane domain, residues 186–210; LBD1, residues 133–144; and LBD2, residues 100–113.

pathways can lead to transcriptional activation of the promoter for interleukin-2. In light of the important role that Lck plays in T-cell growth and differentiation, it is reasonable to suspect that the interaction of the Herpesvirus saimiri Tip protein with Lck plays a role in the induction of T-cell disease by the virus.

Lck is a 56-kDa cytoplasmic tyrosine protein kinase that is anchored to the plasma membrane through a fatty-acylated amino terminus (Fig. 1) (Paige *et al.,* 1993; Yurchak and Sefton, 1995). It contains an SH3 domain that binds to polyproline type II helices (Yu *et al.,* 1994) and an SH2 domain that binds to sites of tyrosine phosphorylation (Eck *et al.,* 1993). These domains have the potential to bind intramolecularly to the linker between the SH2 domain and the catalytic domain, and to phosphorylated tyrosine 505 (Sieh *et al.,* 1993), respectively. Such intramolecular binding inhibits the kinase. It is likely that both the SH3 and SH2 domains also interact with other proteins that function as regulators or effectors of Lck. Lck is activated by phosphorylation of tyrosine 394 (Hardwick and Sefton, 1995), which is found in the activation loop of the catalytic domain, and inhibited by phosphorylation of tyrosine 505 (Amrein and Sefton, 1988; Marth *et al.,* 1988), which is found in a small, noncatalytic domain at the carboxyl-terminus of the protein.

Tip appears to be anchored to cellular membranes by a C-terminal transmembrane domain (Fig. 1) (Lund *et al.,* 1995). It contains two domains that are known to be important for binding Lck (Jung *et al.,* 1995a,b; Lund *et al.,* 1996), one of which is a proline-rich region (residues 132–141) that has been termed the SH3B domain. Here we refer to this domain as Lck-binding domain 1 (LBD1) to avoid confusion with the SH3 domain of Lck (Fig. 1). The other domain required for optimal binding is comprised of residues 104–113 and has some sequence identity to residues 488–497 in the αI helix at the carboxyl-terminal end of the Lck catalytic domain (Biesinger *et al.,* 1995). This domain in Tip has been referred to as the CSKH domain (Fig. 1). Here we refer to this domain as Lck-binding domain 2 (LBD2) (Fig. 1) to avoid confusion with the Csk tyrosine kinase that is a negative regulator of Lck activity (Bergman *et al.,* 1992). Tip contains no other identifiable protein–protein interaction domains or domains with obvious catalytic function.

In the present study, we have sought to identify the mechanism by which the HVS protein Tip from strain 484C associates with and modifies the activity of Lck. We demonstrate that the binding between Lck and Tip involves two domains of both proteins. We show that each binding domain of Tip is involved in increasing the activity of Lck. Our results further demonstrate that, in the absence of other viral proteins, Tip is sufficient to induce significant transcriptional activation in T cells.

RESULTS

There are two Tip-binding regions in Lck

The HVS Tip protein is known to bind to the Lck SH3 domain (Jung *et al.,* 1995b). We wished to know whether the SH3 domain was the sole site in Lck to which Tip bound. Because Tip is difficult to express stably at easily detectable levels, we addressed this question by transient coexpression of Tip and Lck in human 293 cells. We observed avid and extensive binding of WT Tip to WT Lck in this system (Figs. 2A and 2B). In this typical experiment, 30% of the Lck and 100% of the Tip were found in a Tip:Lck complex. Coexpression of Lck with Tip led to the appearance of two additional forms of the Tip protein that had greater apparent molecular weights than did the species of Tip seen in cells expressing only Tip. These novel species are both tyrosine phosphorylated (our unpublished data), and the additional phosphorylation may explain their reduced gel mobility. The extensive phosphorylation of Tip raised the possibility that Lck could bind to sites of tyrosine phosphorylation in Tip through its SH2 domain. Such an interaction, however, is clearly not essential for binding (see below).

To ask whether the SH3 domain of Lck was required for complex formation, we coexpressed Tip with a form of Lck lacking the entire SH3 domain, ΔSH3Lck (Fig. 1) (Reynolds *et al.,* 1992). To detect a complex between Tip and ΔSH3Lck, we assayed for the presence of Lck by incubation of immunoprecipitates *in vitro* with radioac-

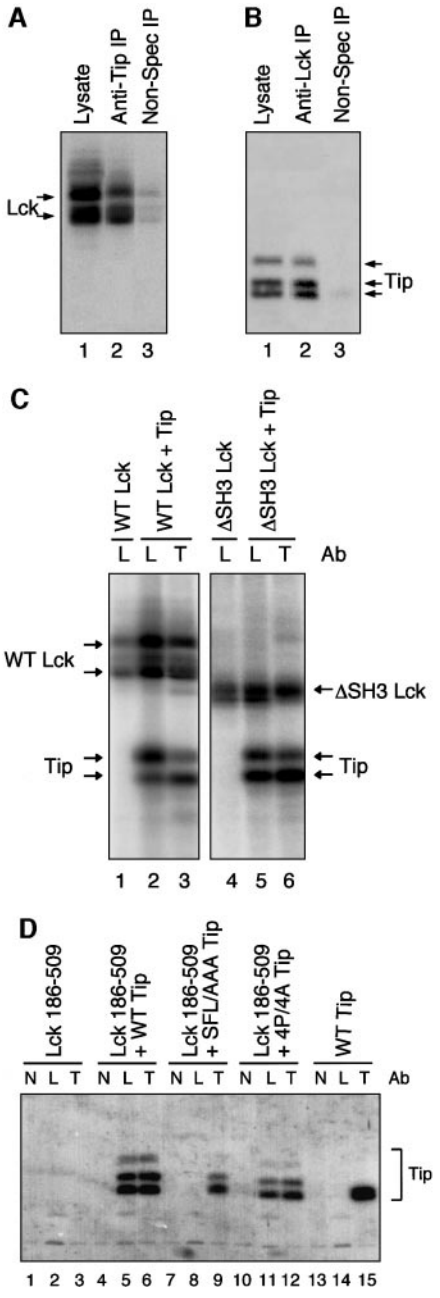

FIG. 2. Tip and Lck bind to each other when coexpressed in 293 cells, and deletion of either the SH3 domain or the amino-terminal third of Lck does not abolish binding. (A and B) Human 293 cells were transiently transfected with plasmids encoding Lck, Tip, or both Lck and Tip. Detergent-soluble lysates were prepared and immunoprecipitation was carried out with antisera to either Lck or Tip under conditions of antibody excess. The immunoprecipitates, and a portion of the cell lysates equal to that used for immunoprecipitation (derived from 1.6×10^4 cells), were subjected to SDS–PAGE. Lck and Tip were quantified by immunoblotting with anti-Lck or anti-Tip antibodies using ^{125}I-protein A as a secondary detection reagent. Immunoprecipitated proteins were quantified using a PhosphorImager. (A) Anti-Lck immunoblot. Lane 1, lysate of cells cotransfected with Lck and Tip; lane 2, anti-Tip immunoprecipitate from cotransfected cells; lane 3, anti-Tip immunoprecipitate from cells transfected only with Lck. (B) Anti-Tip immunoblot. Lane 1, lysate of cells cotransfected with Lck and Tip; lane 2, anti-Lck immunoprecipitate from cotransfected cells; lane 3, anti-Lck immunoprecipitate from cells transfected only with Tip. (C) Human 293 cells were transfected with plasmids encoding either WT Lck, both WT Lck and Tip, ΔSH3Lck, or both ΔSH3Lck and Tip. Immunoprecipitation was carried out with either anti-Lck or anti-Tip antibodies, and the immunoprecipitates were incubated with [γ-^{32}P]ATP *in vitro*. Samples were subjected to SDS–PAGE, and labeled proteins were detected using a PhosphorImager. WT Lck, ΔSH3Lck, and Tip are indicated with arrows; L, anti-Lck antiserum; T, anti-Tip antiserum. Lane 1, anti-Lck immunoprecipitate from cells transfected with WT Lck; lane 2, anti-Lck immunoprecipitate from cells transfected with WT Lck and Tip; lane 3, anti-Tip immunoprecipitate from cells transfected with both WT Lck and Tip; lane 4, anti-Lck immunoprecipitate from cells transfected with ΔSH3Lck; lane 5, anti-Lck immunoprecipitate from cells transfected with ΔSH3Lck and Tip; lane 6, anti-Tip immunoprecipitate from cells transfected with ΔSH3Lck and Tip. (D) Human 293 cells were transfected with plasmids encoding Myc-tagged Lck 186–509, both Myc-tagged Lck 186–509 and WT Tip, both Myc-tagged Lck 186–509 and SFL/AAA Tip, both Myc-tagged Lck 186–509, or WT Tip alone. Immunoprecipitation was carried out with rabbit anti-Tip antiserum, mouse monoclonal anti-Myc antibodies, or a preimmune rabbit serum. Immunoprecipitates from 4×10^4 cells were loaded in each lane. Immunoprecipitated Tip was detected by immunoblotting with anti-Tip antibodies using ^{125}I-protein A as a detection reagent and a PhosphorImager for detection and quantification. The three Tip bands are indicated with a bracket. The DNAs used for transfection are indicated at the top. N denotes samples prepared with a nonspecific antibody. L denotes samples where Lck was immunoprecipitated with anti-Myc tag antibodies. T denotes samples prepared with anti-Tip antibodies. Lane 1, Lck 186–509 only, nonspecific antibody; lane 2, Lck 186–509 only, anti-Myc antibody; lane 3, Lck 186–509 only, anti-Tip antiserum; lane 4, Lck 186–509 and WT Tip, nonspecific antibody; lane 5, Lck 186–509 and WT Tip, anti-Myc antibody; lane 6, Lck 186–509 and WT Tip, anti-Tip antiserum; lane 7, Lck 186–509 and SFL/AAA Tip, nonspecific antibody; lane 8, Lck 186–509 and SFL/AAA Tip, anti-Myc antibody; lane 9, Lck 186–509 and SFL/AAA Tip, anti-Tip antiserum; lane 10, Lck 186–509 and 4P/4A Tip, nonspecific antibody; lane 11, Lck 186–509 and 4P/4A Tip, anti-Myc antibody; lane 12, Lck 186–509 and 4P/4A Tip, anti-Tip antibody; lane 13, WT Tip only, nonspecific antibody; lane 14, WT Tip only, anti-Myc antibody; lane 15, WT Tip only, anti-Tip antiserum.

tive ATP in a protein kinase reaction buffer. The ΔSH3Lck protein phosphorylated both itself and Tip *in vitro* (Fig. 2C). ΔSH3Lck was readily detected in immunoprecipitates prepared with anti-Tip antisera from cells cotransfected with ΔSH3Lck and Tip expression vectors (Fig. 2C). Similarly, Tip was apparent in the immunoprecipitates prepared with anti-Lck antisera from the same cells. Only the two tyrosine phosphorylated species of Tip were labeled during the *in vitro* reaction. The fastest migrating species seen in Fig. 2B is not tyrosine phosphorylated *in vivo* or *in vitro.* Quantitative analysis of the extent of complex formation in this experiment using immunoblotting with anti-phosphotyrosine antibodies (data not shown) revealed that 100% of tyrosine-phosphorylated Tip was bound to WT Lck, whereas 44% of tyrosine-phosphorylated Tip was bound to ΔSH3Lck. Conversely, 40% of WT Lck was bound to Tip, whereas 13% or ΔSH3Lck was bound to Tip. This experiment established that the SH3 domain of Lck was not essential for complex formation between the two proteins and that extensive binding of Lck to Tip occurred in its ab-

sence. This suggested the existence of a second Tip binding site in Lck.

To further localize the second binding site in Lck, we tested the ability of Tip to bind to a fragment of Lck, consisting of residues 186–509, that had been tagged at its amino terminus with six Myc tags (Fig. 1). This deleted form lacks the unique amino-terminal membrane-binding domain, the SH3 domain, and the majority of the SH2 domain. Clear binding of Tip to this fragment of Lck was observed (Fig. 2D). The binding of the Myc-tagged 186–509 fragment of Lck to Tip is notable, in that this form of Lck lacks a membrane-binding domain. Additionally, the binding of this fragment shows that a functional SH2 domain is not required for complex formation. The binding of Tip to this fragment established that there was a Tip-binding site in the carboxyl-terminal half of Lck.

To define the sequences in Tip involved in binding to the carboxyl-terminus of Lck, we examined the ability of mutants of Tip lacking LBD1 or LBD2 to bind to this fragment of Lck. To inactivate LBD1 and create a mutant containing only functional LBD2, we converted prolines 135, 138, 139, and 141 to alanine (4P/4A Tip, Fig. 1). This mutation abolished binding to Tip to a GST fusion protein containing the Lck SH3 domain (data not shown). This mutant Tip protein bound to the carboxyl-terminal fragment of Lck as well as did wild-type Tip (Fig. 2D). LBD1 of Tip, therefore, apparently has no role in binding to Lck in the absence of the Lck SH3 domain. Residues 108–110 in Tip are present in LBD2 and have been shown previously to be required for efficient binding to Lck (Jung *et al.,* 1995a). Mutation of these residues to alanine (SFL/AAA Tip, Fig. 1) abolished detectable binding of Tip to the 186–509 fragment of Lck (Fig. 2D). The ability of wild-type Tip to bind to carboxyl-terminal half of Lck thus appeared to be due to LBD2.

Properties of the Tip protein containing only one functional Lck-binding domain

To study the interaction of Tip with Lck in more detail, we measured the ability of Tip containing only one functional Lck-binding domain to bind to WT Lck. Complexes of Lck and Tip were isolated by immunoprecipitation in a buffer containing 1% NP-40 detergent from transiently transfected 293 cells. We always observe some nonspecific precipitation of both Lck and Tip in this mild lysis buffer. To quantify the nonspecific precipitation accurately, control precipitates were prepared with antibodies to either Lck or Tip from lysates of cells expressing only Tip or Lck, respectively (Figs. 3A and 3B), or with nonspecific serum from doubly transfected cells (data not shown). The nonspecific precipitation observed under these conditions was quantified and subtracted from the specific precipitation to yield accurate estimates of the extent of complex formation. Mutant Tip containing either only the proline-rich LBD1 or only LBD2 bound to WT Lck, albeit to a reduced extent (Figs. 3A and 3B). In this experiment, 56% of WT Tip was bound to Lck, whereas 27% of the Tip lacking LBD1 and 20% of the Tip lacking LBD2 was found in a complex with Lck. No detectable binding to Lck was observed with a mutant of Tip lacking both LBD1 and LBD2 (data not shown).

We also tested the ability of the mutant Tips lacking either a functional LBD1 or LBD2 to stimulate tyrosine phosphorylation of cellular proteins (Fig. 3D). Expression of Tip alone had no effect on tyrosine phosphorylation in the cell lysate. The expression of Lck led to a small, but detectable, increase in protein tyrosine phosphorylation, much of which was due to the tyrosine phosphorylation of Lck itself. Coexpression of WT or either mutant Tip with Lck stimulated tyrosine phosphorylation of cellular proteins considerably (Fig. 3D). The extent of protein phosphorylation induced by the mutant Tip proteins, however, was reduced relative to that induced by the WT Tip protein (Fig. 3D). A mutant Tip protein carrying mutations in both LBD1 and LBD2 exhibited little ability to elevate tyrosine phosphorylation of cellular proteins (Fig. 3D).

Activity of Tip mutants in T cells

We next tested the ability of the mutant Tip proteins to stimulate transcription in transiently transfected Jurkat T cells. Because Tip has been shown to stimulate Stat1 and Stat3 DNA binding (Lund *et al.,* 1997, 1999), we first examined the ability of the mutant Tip proteins to induce increased transcription from a reporter that has been shown previously to respond to the activation of Stat3 (Turkson *et al.,* 1998). Because Tip was not detectable in these transfected cells, differences in transfection efficiency were corrected for by the inclusion in each transfection of a plasmid expressing *Renilla* luciferase from an actin promoter. The data shown here are from a single experiment and are representative of the results of seven experiments with Stat-responsive reporters. WT Tip induced a fivefold increase in expression of luciferase driven by this reporter (Fig. 4). This was considerably greater than the 40% stimulation induced by activated Lck (F505Lck). Both LBD1- and LBD2-mutant Tip proteins stimulated expression from this reporter. The mutant containing only LBD2 (4P/4A Tip) consistently showed more activity (75% of that of wild-type Tip) than the mutant containing only LBD1 (SFL/AAA Tip). Doubly mutant Tip containing both a mutant LBD1 and a mutant LBD2 exhibited no activity in this assay (Fig. 4).

In contrast to the Stat3-responsive reporter, both WT Tip and activated Lck stimulated transcription from a cotransfected reporter construct driven by a promoter containing NF-AT (Nuclear factor of activated T-cells) sites, inducing a six- to 10-fold increase in transcription (Fig. 4). Strikingly, neither the LBD1 nor the LBD2 mutant

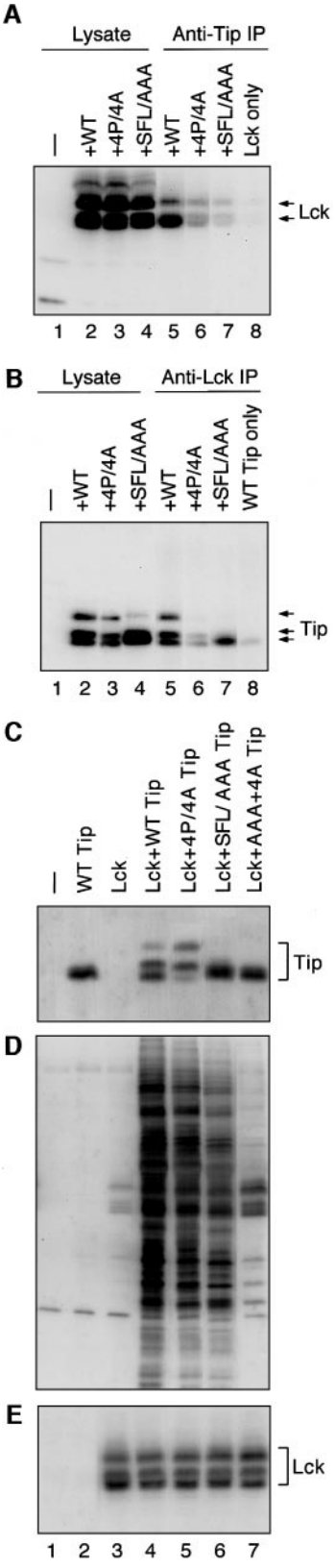

FIG. 3. Both SFL/AAA Tip and 4P/4A Tip bind to and activate Lck. Human 293 cells were transfected with plasmids encoding Lck, WT Tip, both Lck and WT Tip, both Lck and SFL/AAA Tip, both Lck and 4P/4A Tip, or both Lck and AAA+4A (doubly mutant). Tip and detergent-soluble lysates were prepared. Immunoprecipitates were prepared with anti-Lck or anti-Tip antiserum in antibody excess. The immunoprecipitates and a portion of the cell lysate equal to that used for immunoprecipitation (from 1.6×10^4 cells) were subjected to SDS–PAGE. Lck and Tip were quantified by immunoblotting with anti-Lck (A) or anti-Tip (B) antibodies respectively, using ^{125}I-protein A as a detection reagent. Lck and Tip are indicated with arrows. (A) Anti-Lck immunoblot. Lane 1, untransfected cell lysate; lane 2, Lck and WT Tip lysate; lane 3, Lck and 4P/4A Tip lysate; lane 4, Lck and SFL/AAA Tip lysate; lane 5, anti-Tip immunoprecipitate from Lck and WT Tip lysate; lane 6, anti-Tip immunoprecipitate from Lck and 4P/4A Tip lysate; lane 7, anti-Tip immunoprecipitate from Lck and SFL/AAA Tip lysate; lane 8, anti-Tip immunoprecipitate from lysate of cells transfected with only Lck DNA. (B) Anti-Tip immunoblot of the samples from the same experiment as in (A). Lane 1, untransfected cell lysate; lane 2, Lck and WT Tip lysate; lane 3, Lck and 4P/4A Tip lysate; lane 4, Lck and SFL/AAA Tip lysate; lane 5, anti-Lck immunoprecipitate from Lck and WT Tip lysate; lane 6, anti-Lck immunoprecipitate from Lck and 4P/4A Tip lysate; lane 7, anti-Lck immunoprecipitate from Lck and SFL/AAA Tip lysate; lane 8, anti-Lck immunoprecipitate from lysate of cells transfected with only Tip DNA. (C, D, and E) Data from a separate experiment analyzing tyrosine phosphorylation induced by the mutant Tip proteins. Tip levels, Lck levels, and tyrosine phosphorylation in cell lysates (from 4×10^4 cells) were quantified by Western blotting analysis with antibodies to Tip, Lck, or phosphotyrosine using ^{125}I-protein A as a detection reagent. Detection was accomplished with a PhosphorImager. The same samples were analyzed in (C), (D), and (E). (C) Anti-Tip immunoblot of cell lysates. (D) Anti-phosphotyrosine immunoblot of cell lysates. (E) Anti-Lck immunoblot of cell lysates. Lane 1, untransfected cells; lane 2, WT Tip alone; lane 3, Lck alone; lane 4, Lck and WT Tip; lane 5, Lck and 4P/4A (LBD2 only) Tip; lane 6, Lck and SFL/AAA (LBD1 only) Tip; lane 7, Lck and AAA+4A Tip.

Tip was able to induce significant NF-AT-dependent transcription in any of 10 experiments.

DISCUSSION

It was established previously that two regions of Tip were involved in the binding of the protein to Lck (Jung *et al.,* 1995a; Lund *et al.,* 1996). We have shown here that these two domains bind to different sites in Lck. The proline-rich LBD1 binds to the SH3 domain in Lck (Fig. 5). LBD2, a region with the sequence EDLQSFLEKY, binds an as-yet-unidentified site in the carboxyl-terminal half of Lck (Fig. 5). Precise mapping of where in the carboxyl-terminal portion of Lck the Tip LBD2 binds will probably come only from determination of the crystal structure of the complex. The binding of Tip through either domain activates Lck detectably *in vivo.* Activation was apparent from analysis of the phosphorylation of cellular proteins in transfected 293 cells expressing both Lck and Tip, and by transient cotransfection of Jurkat T cells with Tip and a reporter construct driven by a Stat-responsive promoter. The biological activity of mutants of Tip lacking either Lck-binding domain, however, was clearly not equivalent to that of the WT protein. This was most apparent from the fact that neither mutant Tip protein exhibited sufficient activity in T cells to stimulate transcription from a reporter containing three NF-AT sites. Additionally, both mutant Tip proteins differ from WT Tip

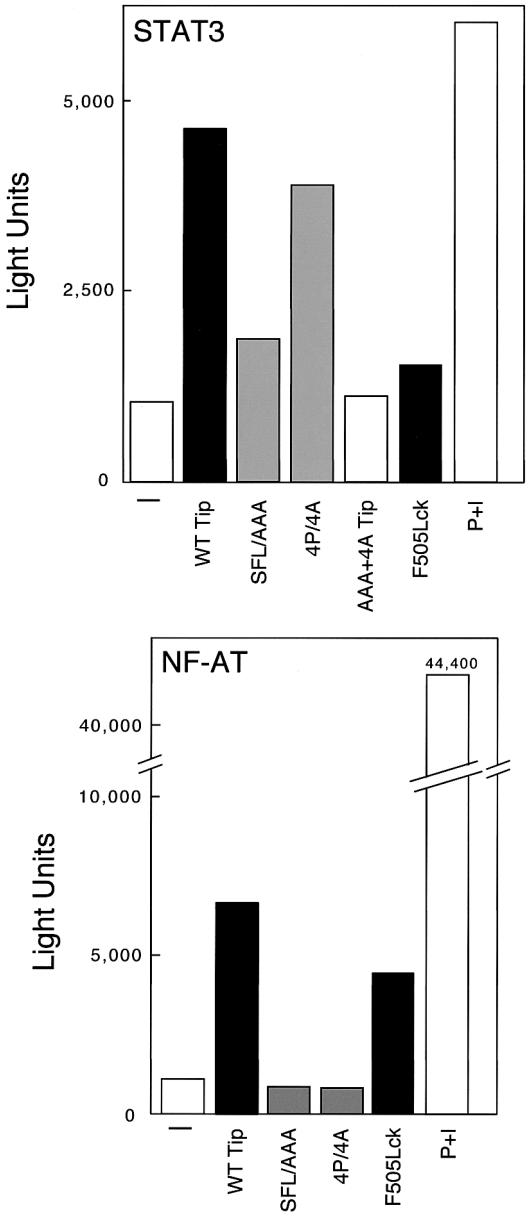

FIG. 4. The ability of Tip mutants to stimulate transcription from Stat3- and NF-AT-responsive reporters in Jurkat cells. Jurkat T antigen cells were transfected with plasmids encoding a Stat3-responsive firefly luciferase reporter (top panel) or an NF-AT-responsive firefly luciferase reporter (bottom panel) together with plasmids encoding WT Tip, SFL/AAA Tip, 4P/4A Tip, doubly mutant AAA+4A Tip, or activated F505 Lck. Cells transfected with only the reporter construct were incubated with 60 nmol phorbol myristyl acetate (PMA) and 1.5 μmol ionomycin (Calbiochem) for 4 h to mimic stimulation of the T-cell antigen receptor. Luciferase levels were quantified as described under Materials and Methods. (—) indicates cells transfected only with the reporter. P+I indicates cells transfected with the reporter and incubated with PMA and ionomycin.

in that they cannot activate Lck detectably in a cell-free system (Hartley *et al.,* 1999). We do not yet know whether intact cells contain a factor that helps stabilize the complex of mutant Tip and Lck, or whether modification of Lck must occur in intact cells to allow activation by the mutant Tip proteins.

The ability of each binding domain to partially activate Lck suggests that activation of Lck by WT Tip has two components that must cooperate to fully activate Lck. The binding of LBD1 to the SH3 domain of Lck probably activates Lck by displacement of the SH3 domain of Lck (Fig. 5). The structure of two Src kinases, Hck and c-Src, has been determined (Sicheri *et al.,* 1997; Williams *et al.,* 1997; Xu *et al.,* 1997). In both structures the SH3 domains of the kinases were found to be bound intramolecularly to a polyproline II helix formed by the linker between the SH2 domain and the catalytic domain. It is thought that this interaction constrains the catalytic domain and thereby inhibits the assumption of a catalytically active conformation. The Nef protein of human immunodeficiency virus is a high-affinity ligand for the SH3 domain of Hck (Lee *et al.,* 1995; Saksela *et al.,* 1995). The addition of Nef to Hck stimulates the kinase activity of Hck (Moarefi *et al.,* 1997). It is likely that this activation results from the binding of Nef to the SH3 domain, displacement of the SH3 domain from the SH2/catalytic domain linker, and removal of conformational constraints. Although the crystal structure of Lck has not yet been determined, it is reasonable to assume that the SH3 domain of Lck can bind intramolecularly to the region surrounding Pro 229 in the SH2/catalytic domain linker (Fig. 5). Such an interaction would be expected to be inhibitory, an idea that is supported by the fact that mutations in this region activate Lck (Gonfloni *et al.,* 1997; Wright *et al.,* 1994). It is likely, therefore, that the binding of the proline-rich LBD1 of Tip to the Lck SH3 domain relaxes the constrained conformation and increases the catalytic activity of Lck (Fig. 5).

How the binding of LBD2 in Tip to the carboxyl-terminal half of Lck stimulates catalytic activity is less obvious. Two possible mechanisms can be considered. The LBD2 domain of Tip has some sequence identity to the αI helix that is found at the carboxyl-terminal end of the Lck catalytic domain. Additionally, the PredictProtein program (Rost, 1996) predicts that this domain in Tip will assume an α-helical conformation. It is possible that, through a mechanism similar to that envisioned for the interaction of LBD1 with the SH3 domain, the LBD2 of Tip binds to the catalytic domain where the αI helix does so normally, displaces the αI helix, and that this perturbation is activating. Consistent with the possibility that an interaction with the αI helix could be activating is the finding that two spontaneous mutations of the c-Src gene that activate the enzyme are found in positions Glu378 and Ile441 (Levy *et al.,* 1986), both of which impinge on the αI helix (Sicheri *et al.,* 1997).

Alternatively, it is conceivable that LBD2 of Tip activates Lck through a mechanism similar to the activation of the cyclin-dependent protein kinases by cyclins. Cyclins bind to CDKs in the small, amino-terminal lobe of the catalytic domain (Jeffrey *et al.,* 1995). This binding induces, or allows, the reorientation of the αC helix. It is

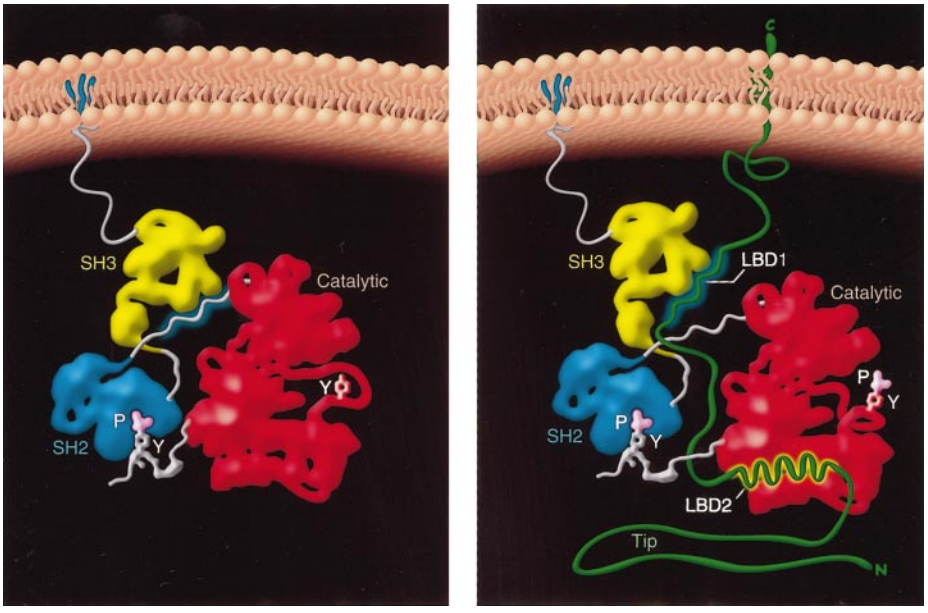

FIG. 5. Structure of Lck and hypothetical model of how Tip binds to Lck. The structures of c-Src and Hck suggest that the SH3 domain of Lck, in the absence of Tip, is bound to the linker between the SH2 domain and the catalytic domain and that the SH2 domain is bound to phosphorylated tyrosine 505. Tyrosine 394 in the activation loop is not phosphorylated in this form of Lck. Tip binds to two sites in Lck. Proline-rich LBD1 binds to the SH3 domain, presumably displacing it and disrupting the intramolecular complex. The LBD2 region of Tip binds to an as-yet imprecisely mapped site in the carboxyl-terminal half of Lck. LBD2 is shown here binding to the large lobe of the catalytic domain. Lck bound to Tip is phosphorylated to an increased extent at tyrosine 394 (Hartley *et al.,* 1999). The increased phosphorylation of this site almost certainly contributes to activation.

formally possible that LBD2 also binds to the small, amino-terminal lobe of the catalytic domain and this binding induces reorientation of the αC helix. Clarification of the actual mechanism of activation of Lck by Tip, however, will come only from structural analysis.

Both Tip and Lck are membrane proteins; however, this property does not play an essential role in their interaction. We found that a form of Lck in which the membrane-binding domain had been deleted and replaced with six Myc tags still bound to Tip (Fig. 2D). Additionally, Tip lacking its carboxyl-terminal membrane anchor activates Lck in T cells (Lund *et al.,* 1999). These data suggest that the affinity of Tip for Lck is sufficiently great that colocalization of the two proteins on cellular membranes is not required for complex formation. Because a majority of Tip undergoes tyrosine phosphorylation when coexpressed with Lck (our unpublished observations), it was possible that the SH2 domain of Lck played a role in complex formation. The fact that the 186–509 fragment of Lck, which lacks the majority of the SH2 domain, binds to Tip (Fig. 2D) demonstrates that the SH2 domain of Lck is certainly not required for binding. It is possible, however, that the SH2 domain stabilizes the complex.

Although the ability of the Tip protein of HVS, strain 488C, to bind to Lck *in vitro* is reduced by mutation of prolines in LBD1 (Duboise *et al.,* 1998b; Jung *et al.,* 1995a), a virus carrying this mutation is reported to still be able to induce lymphomas *in vivo* (Duboise *et al.,* 1998b). This observation is consistent with our finding that mutation of prolines in LBD1 of the Tip from strain 484C reduces, but does not eliminate, the ability of the protein to stimulate protein phosphorylation and transcription from a Stat-responsive promoter. It has been reported that the mutation of LBD1 of strain 488C abolishes detectable binding of Tip to Lck in leukemic cells *in vivo* (Duboise *et al.,* 1998b), and this has been interpreted to mean that the binding of Tip to Lck is not required for disease induction. In contrast, we found that complexes between Lck and Tip with a mutated LBD1 can be detected, albeit at a lower level than those seen with the WT protein, in transiently transfected 293 cells. Because Tip is expressed at extremely low levels in virally infected leukemic cells, a complex between Tip and Lck with reduced stability might well be extremely difficult to detect.

We note that, whereas we find the Tip protein from strain 484C of Herpesvirus saimiri to activate Lck, the closely related Tip protein from strain 488C of Herpesvirus saimiri is reported to inhibit Lck (Jung *et al.,* 1995b). Additionally, mutation of Tyr114 in Tip from strain 488C increases both the binding of Tip to Lck and the inhibition of Lck (Guo *et al.,* 1997). The question of why these two closely related viral transforming proteins have apparently completely opposite effects on Lck remains unanswered.

The Tip protein of HVS is similar to two other viral proteins that target Src kinases, the Nef protein of HIV and the middle T antigen of polyoma virus. Like Nef, Tip contains a domain that binds to an SH3 domain; how-

ever, Tip is not simply an SH3 domain ligand. Like middle T antigen (Dunant *et al.,* 1996), Tip contains a domain that interacts with the carboxyl-terminal half of Src kinases. Thus, Tip is unique in that it contains two Src kinase binding domains, both of which are required for full activity. Viral transforming proteins often function as mimics of cellular proteins, which may also be the case for Tip. There may well exist in T cells a cellular protein that is analogous to Tip in functioning as an activator of Lck through binding interactions with both the SH3 domain and the catalytic domain. Such a protein could play an important role in T-cell responses. There are suggestions that the β-arrestin protein may activate the c-Src kinase through binding interactions (Luttrell *et al.,* 1999; Miller *et al.,* 2000) that have some similarity to those by which Tip activates Lck. It is clearly of considerable interest to determine whether there is a normal cellular protein that is a functional homolog of Tip.

MATERIALS AND METHODS

Antibodies and cell lines

Polyclonal antiserum to the Herpesvirus saimiri Tip protein was raised in rabbits using a GST fusion protein containing amino acids 1–84 of Tip-484C. Specificity of this antisera was confirmed by comparison to a previously characterized anti-Tip sera (Lund *et al.,* 1997). The rabbit anti-Lck (Hurley and Sefton, 1989) and rabbit anti-phosphotyrosine (Kamps and Sefton, 1988) antibodies have been described previously. The 9E10 monoclonal anti-Myc antibody (Evan *et al.,* 1985) was kindly provided by Jill Meisenhelder and Tony Hunter (The Salk Institute). Human 293 cells were grown in DMEM supplemented with either 5% fetal calf serum (Intergen, Purchase, NY) and 5% iron-supplemented calf serum (Gemini Bio-Products, Calabasas, CA) or 10% iron-supplemented calf serum and penicillin/streptomycin. Jurkat cells that express the large T antigen of SV40 virus (Northrop *et al.,* 1993) were kindly provided by Jack Bui and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin.

Plasmid expression vectors

The Tip and Lck constructs are diagrammed in Fig. 1. To inactivate LBD1, PCR mutagenesis was used to change prolines 135, 138, 139, and 141 to alanine (4P/4A Tip, Fig. 1). To inactivate LBD2, we mutated three residues, Ser108, Phe109, and Leu110, to alanines (SFL/AAA Tip, Fig. 1). All mutations were confirmed by sequencing. For transfection of 293 cells, all Tip constructs were inserted into the pCEP4 vector (Invitrogen, Carlsbad, CA). For T-antigen Jurkat cell transfection, Tip constructs were moved to the pRc/RSV vector (Invitrogen).

The ΔSH3Lck construct, derived from murine Lck and previously described (Reynolds *et al.,* 1992), was expressed using the pCMX vector (Umesono *et al.,* 1991). Lck 186–509 was generated by subcloning of an *Xho*I–*Eco*RI fragment from ΔSH2F505Lck in the plasmid LXSN (Reynolds *et al.,* 1992) into CS3+MT (a gift of Dr. Jon Cooper, Fred Hutchinson Cancer Research Center). This vector appends six Myc tags to the amino terminus. Wild-type (WT) Lck was expressed in 293 cells using the pCEP4 vector. F505 Lck, a constitutively activated form of Lck (Amrein and Sefton, 1988), was expressed in T-antigen Jurkat cells using either the SRα3 vector or the pRcRSV vector. Two firefly luciferase reporter constructs, one containing a promoter consisting of three copies of an NF-AT site and a fragment of the human interleukin-2 promoter (Northrop *et al.,* 1993) (a gift from J. Bui), the other containing seven copies of a Stat3-responsive element from the C-reactive protein gene (Turkson *et al.,* 1998) (a gift from Richard Jove, Moffitt Cancer Center), were used. A plasmid encoding *Renilla* luciferase under the control of the β-actin promoter was a gift of Christopher Herold (UCSD).

Protein expression, immunoprecipitation, and immunoblotting

Human 293 cells were seeded at 4.5×10^5 cells per 60-mm gelatin-coated petri dishes the day prior to transfection, which was performed using a standard calcium-phosphate precipitation technique. At 48 h posttransfection the cells were lysed in 600 μL of 1% Nonidet-P40, 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1 mM sodium vanadate, 2 mM EDTA, and 10 KIU/ml Aprotinin. Lysates were then clarified by centrifugation at 10,000 *g* for 15 min at 4°C. Cleared lysates were incubated for 40–60 min at 4°C with 1 μL of the specified primary antisera. Pansorbin cells (Calbiochem, La Jolla, CA) were added, and incubations continued for a further 20–60 min. Immune complexes were then washed three to five times. Immunoprecipitates and cell lysates were subjected to SDS–PAGE and transferred to Protran (Schleicher and Schuell, Keene, NH) or Immobilon-P (Millipore, Bedford, MA) for immunoblotting. Primary antibodies were detected with 10 μCi ^{125}I-protein A (ICN, Irvine, CA).

Assays of kinase activity

After washing, precipitates were resuspended in 10 μL of kinase assay buffer (40 mM PIPES, pH 7.0, and 10 mM $MnCl_2$). An additional 10 μL of kinase buffer was added containing 5 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL), and samples were incubated at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of a cold 50 mM Tris (pH 7.8), 150 mM NaCl, and 0.01% NP-40 solution; samples were pelleted; and the supernatant containing unincorporated ^{32}P was discarded.

Luciferase assays

T-antigen Jurkat cells were transfected with 1 μg of luciferase reporter and 3 μg of an expression vector containing Tip DNA, Lck DNA, or no insert. Transfection was performed with DMRIE-C using the protocol recommended by the supplier (Life Technologies, Gaithersburg, MD). A 200-ng sample of a reporter construct expressing *Renilla* luciferase under control of the β-actin promoter was included in each transfection. After 48 h approximately 4×10^6 transfected cells were washed once in cold PBS (without Ca^{2+} or Mg^{2+}) followed by resuspension in 100 μL lysis buffer (0.1 M potassium phosphate, pH 7.8, 1% Triton X-100, 1 mM DTT, and 2 mM EDTA). Cells were incubated for 20 min at 4°C before pelleting to remove insoluble material. Supernatants were removed and placed on ice until analyzed. Aliquots (30 μL) from each sample were mixed with 100 μL assay buffer (30 mM Tricine, 3 mM ATP, 15 mM $MgSO_4$, and 10 mM DTT, final pH 7.8) immediately prior to reading in a Berthold Lumat LB 9507 luminometer. The reactions were initiated by injection of 100 μL of freshly prepared 1 mM D-luciferin (pH 6.1–6.5) into each sample. The luciferase assay was quenched with Stop & Glo (Promega, Madison, WI) and the activity of the *Renilla* enzyme was measured. This value was used as a measure of transfection efficiency and was used to normalize the firefly luciferase data.

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